Binding of Calcium to Individual γ-Carboxyglutamic Acid Residues of Human Protein C†

Tracey L. Colpitts, Mary Prorok, and Francis J. Castellino*

Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

Received July 26, 1994; Revised Manuscript Received November 10, 1994®

ABSTRACT: Selectively labeled polypeptides comprising the γ-carboxyglutamic acid (Gla) domain (GD) and helical stack (HS) regions of human protein C (PC), and consisting of amino acid residues 1−47, have been chemically synthesized and their Ca^{2+} binding properties assessed by [^{13}C]-NMR methods. A total of nine such polypeptides have been studied, each containing one of the Gla residues fully enriched with [^{13}C] at its two γ-carboxylate carbon atoms. Additions of Ca^{2+} resulted in readily measurable [^{13}C] chemical shifts, titrations of which were used to obtain apparent dissociation constants for each Gla residue in the presence of all other such residues. The Ca^{2+} titration data obtained on each of the nine polypeptides showed that Gla residues 6, 16, 25, and 26 were involved in the higher affinity Ca^{2+} binding sites, whereas the remaining Gla residues, *viz.,* 7, 14, 19, 20, and 29, coordinated Ca^{2+} more weakly. The results are consistent with conclusions drawn from functional studies obtained with site-directed mutations of individual Gla residues and with the structural model of the GD/HS of human PC. In these cases, Gla residues 6, 16, and 26 served as coordination loci for internally located Ca^{2+} ions, and GD-related Ca^{2+}- and PL-dependent properties of PC and activated PC were dependent on the integrity of these Gla residues.

PC,[1] one of the vitamin K-dependent blood coagulation proteins, is the zymogen of the serine protease APC, an enzyme with anticoagulant activity (Kisiel et al., 1977; Vehar & Davie, 1980). In order to function properly, PC and APC require, among other more common posttranslational processing steps, events that result in γ-carboxylation of all nine Glu residues in its amino-terminal GD. This domain is believed to function by providing numerous Ca^{2+} binding sites to the proteins (Nelsestuen & Suttie, 1972; Bajaj et al., 1975; Henriksen & Jackson, 1975; Amphlett et al., 1979, 1981a,b; Strickland & Castellino, 1980; Bajaj, 1982). Occupation of these sites by Ca^{2+}, and some other related cations, has been proposed to induce the Ca^{2+}-dependent conformation required for binding of PC and related proteins to acidic PL (Borowski et al., 1986b; Liebman et al., 1987; Liebman, 1993), an event of functional consequence for both PC and APC. Attainment of the GD-specific Ca^{2+}-induced conformation for different proteins of this class can normally be probed by intrinsic fluorescence quenching (Nelsestuen, 1976; Prendergast & Mann, 1977; Strickland & Castellino, 1980; Astermark et al., 1991; Zhang & Castellino, 1992) and by interactions with antibodies specific for the Ca^{2+}-directed conformation of the GD (Keyt et al., 1982; Borowski et al., 1986a; Wakabayashi et al., 1986; Liebman et al., 1987; Church et al., 1989a; Zhang & Castellino, 1992; Liebman, 1993). A minimum of two classes of Ca^{2+} binding sites have been found in the GD, a small number of tighter sites that are relatively nonspecific for the cation (Keyt et al., 1982; Borowski et al., 1986a; Wakabayashi et al., 1986; Liebman et al., 1987; Church et al., 1989b; Liebman, 1993) as well as a weaker class of multiple sites that are more specific for Ca^{2+} (Nelsestuen, 1976; Borowski et al., 1986a; Liebman et al., 1987; Liebman, 1993). Regarding the number of Ca^{2+} ions that are contained in the GD, the X-ray crystal structure of the Ca^{2+}/GD complex of prothrombin fragment 1 has been published (Soriano-Garcia et al., 1992) and contains three internal and four additional surface-exposed Ca^{2+} ions.

On the basis of Ca^{2+} binding and other conformational analyses, we have previously shown that a synthetic polypeptide of PC containing the GD and its trailing nine-residue HS provides an excellent model to investigate Ca^{2+} binding to the same regions of intact PC (Colpitts & Castellino, 1994). This finding provides a unique opportunity to correlate the Ca^{2+}-dependent functional properties, revealed through genetic engineering approaches, with Ca^{2+} binding to individual Gla residues. The intact proteins cannot be employed to establish such relationships because of the impossibility of separating the contributions of individual Gla residues to cation binding in wild-type or mutant proteins. Therefore, a manner of measuring Ca^{2+} binding

† Supported by Grant HL-19982 from the National Institutes of Health and the Kleiderer/Pezold Family endowed professorship (to F.J.C.).

* To whom to address correspondence.

® Abstract published in *Advance ACS Abstracts,* February 1, 1995.

[1] Abbreviations: PC, protein C; APC, activated protein C; Gla, γ-carboxyglutamic acid; GD, Gla domain, *viz.,* the γ-carboxyglutamic acid-rich region of PC (amino acid residues 1−38); HS, the helical stack region of protein C (amino acid residues 39−47); EGF-1, the first epidermal growth factor homology region of PC consisting of amino acid residues 47−92; PL, 60%/40% (w/w) sonicated dispersion of chicken egg phosphatidylcholine (PhC)/bovine brain phosphatidylserine (PhS); [$C_{50,Ca}$-PL], the total Ca^{2+} concentration required to induce binding to phospholipid vesicles of 50% of the protein molecules at a constant concentration of protein; [$C_{50,P}$-PL], the total protein concentration required to induce binding to phospholipid vesicles of 50% of the protein molecules at a constant concentration of Ca^{2+}; Fmoc, 9-fluorenylmethyloxycarbonyl; *O*-tBu, *tert*-butyl ester; tBoc, *tert*-butyloxycarbonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Bum, *tert*-butyloxymethyl; *O*-Pfp, pentafluorophenyl ester; FPLC, fast protein liquid chromatography; $DodSO_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; [^{13}C-Gla^x]-GD/HS, the particular Gla residue (x) selectively labeled with [^{13}C] in the γ-carboxylates of the 48-mer comprising the amino acid sequences of the Gla domain and helical stack of human protein C; wt, wild type.

0006-2960/95/0434-2424$09.00/0 © 1995 American Chemical Society

to individual Gla residues in the presence of all other such residues was required. Due to our previous finding of the appropriateness of the GD/HS synthetic polypeptide as a model of Ca^{2+}/GD binding in PC, we attempted an isotopic enrichment strategy to functionally isolate individual Gla residues in the wild-type 47-mer polypeptide. A report of the results of this study is presented herein.

MATERIALS AND METHODS

Synthesis of N^{α}-Fmoc-(γ,γ'-di-O-tBu)-L-Gla-OH. The derivatized Gla was synthesized by previously described procedures (Marki et al., 1977), beginning with (1,3-di-*O*-tBu) malonate or [1,3-di-^{13}C]-(1,3-di-*O*-tBu) malonate. This latter material was prepared by an acid-catalyzed transesterification of [1,3-di-^{13}C]malonate with CH_3COO-tBu (Colpitts & Castellino, 1993).

Peptide Synthesis. The following N^{α}-Fmoc-L-amino acids were employed for peptide synthesis: Ala-OH, Asp(*O*-tBu)-OH, Phe-OH, Ile-OH, Leu-OH, Asn-(*O*-Pfp), Gln-(*O*-Pfp), Ser(*O*-tBu)-OH, and Thr(*O*-tBu)-OH (Sigma Chemical Co., St. Louis, MO), Lys(ϵ-*N*-tBoc)-OH, His(Bum)-OH, and Arg-(Pmc)-OH (Calbiochem, La Jolla, CA), and Cys(*S*-thio-tBu)-OH (Bachem, Torrance, CA).

The peptides were synthesized by solid-state methods on a 0.1-mmol scale using an Excell peptide synthesizer (Milligen/Biosearch, Burlington, MA). The products were deprotected and cleaved from the resin as described previously (Colpitts & Castellino, 1993).

Peptide Purification. After removal from the resin and filtration, the peptides were precipitated by addition of diethyl ether and redissolved in 5% NH_4OH. For deprotection of Cys residues and formation of the Cys^{17}–Cys^{22} disulfide bond, the pH of the solution was adjusted to 9.0 with NaOH and 1 g of reduced dithiothreitol was added. Removal of the tBu mercaptan protecting group was accomplished by stirring the peptide under N_2 at room temperature. Formation of the internal disulfide bond was accomplished by dialyzing a dilute solution of the peptide (<1 mg/mL) against a buffer composed of 25 mM Tris-HCl, pH 7.4, at 4 °C for 24 h.

Purification was accomplished by FPLC using a MonoQ (10/10) column (Pharmacia, Piscataway, NJ), equilibrated with 25 mM Tris-HCl, pH 7.5. The peptide was eluted after application of a NaCl gradient. The resulting material was desalted on a column of Sephadex G25 that was equilibrated and eluted with H_2O and lyophilized.

Binding of Ca^{2+} to the Peptides by [^{13}C]-NMR. Titrations with Ca^{2+} of the chemical shifts of [^{13}C]-labeled Gla residues in the synthetic peptides were performed as described (Colpitts & Castellino, 1993, 1994). $K_{d,app}$ values were calculated from the titration of the [^{13}C] chemical shifts as a function of the free [Ca^{2+}]. These latter values were determined at each point during the titration by a Ca^{2+}-specific electrode (Colpitts & Castellino, 1993).

Quasielastic Light Scattering. PL vesicles containing 60/40 (w/w) bovine brain PhC/chicken egg PhS (Sigma Chemical Co.) were prepared as described earlier (Beals & Castellino, 1986). The PL concentration was determined as total organic phosphate (Lowry & Lopez, 1946) using a conversion factor of 25 (w/w) to obtain the weight concentrations of the PL vesicles.

Our methods for determining binding parameters of proteins to PL by 90° relative light scattering have been described in detail earlier (Zhang & Castellino, 1993).

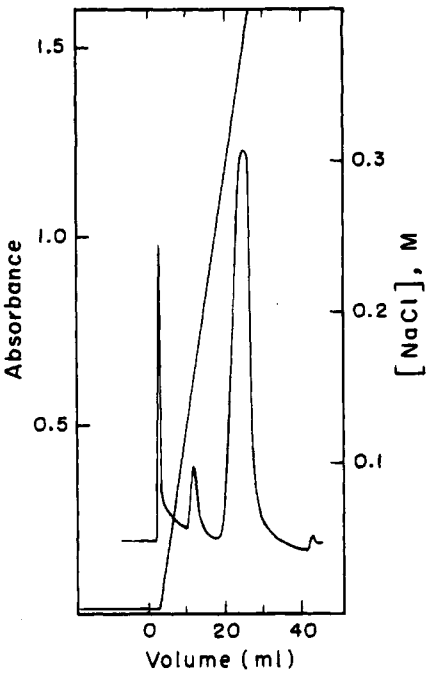

FIGURE 1: Analytical FPLC of wild-type GD/HS on MonoQ 5/5 resin. The buffer was 25 mM Tris-HCl, pH 7.5 at room temperature. The flow rate was 1 mL/min, and elution was accomplished with a NaCl gradient. The major peak was identified as the desired product.

Molecular Model of the Gla Domain and Trailing Helical Stack of Human PC. The three-dimensional structure of the GD/HS region of PC was approximated from these same regions of the structure of bovine prothrombin fragment 1, which has been derived from crystallographic analysis. The general method involved use of an Evans and Sutherland interactive computer graphics display driven by FRODO (Tulinsky et al., 1988) and has described in a previous publication (Christiansen et al., 1994).

Analytical Methods. Our exact procedures for performance of amino acid analysis and amino acid sequencing of peptides (Chibber et al., 1990), as well as for determination of their Gla contents (Zhang & Castellino, 1990), were as described.

RESULTS

Each of the nine [^{13}COOH]-Gla-containing peptides was synthesized using automated solid-state methodology described previously (Colpitts & Castellino, 1993, 1994). An example of the FPLC behavior of the resulting 47-mer peptides is illustrated by that of the wt GD/HS peptide in Figure 1. Few special difficulties were encountered in purification, and quantities of approximately 200 mg were obtained, representing a final yield of 30%. Oxidation of Trp^{41} during cleavage from the resin presented some problems, and prevention of this was accomplished by addition of indole (10%)/thiophenol (10%)/dithioethane (10%) to the 50% trifluoroacetic acid/10% anisole mixture in CH_2Cl_2. No problems were encountered in formation of the required disulfide bond between Cys^{17} and Cys^{22} under the described conditions, and this oxidation was shown to be complete by titrations with Ellman's reagent during the period of oxidation. Amino acid compositions of the nine peptides revealed that all contained the correct proportions of amino acid residues, within experimental error. Gla

contents ranged from 8.3 to 10.4 ± 1.0 residues/mol of peptide. This is within the acceptable range of the theoretical value of 9.0 residues/mol of peptide. Amino acid sequencing through the first five amino acid residues provided the expected results for all peptides, demonstrating that the syntheses reached completion. All peptides showed single bands on $DodSO_4$/PAGE, at approximately the same molecular weights. These results indicated that the expected peptides were obtained at high degrees of purity.

As a quality control measure, each peptide was subjected to acidic PL binding experiments, since this represents the best overall measure of the integrity and similarities of their Ca^{2+} binding properties. Titrations with Ca^{2+} of these interactions were determined with all nine peptides synthesized, and [$C_{50,Ca}$-PL] values ranged between 1.1 and 1.3 mM for the entire set of polypeptides. These values are in agreement with the same parameter determined earlier for this same wt GD/HS 47-mer (Colpitts & Castellino, 1994).

The selectively labeled peptides were then employed for titration with Ca^{2+} with the purpose of identifying the $K_{d,app}$ of each Gla residue in this peptide. The binding constants are considered apparent binding constants because the macroscopic $[Ca^{2+}]_{free}$ is dictated not only by binding to the labeled Gla but also by virtue of the binding properties of all other Gla residues in the polypeptide. Titrations were accomplished by monitoring the chemical shifts as a function of the $[Ca^{2+}]_{free}$. To determine the $[Ca^{2+}]_{free}$ at each point, an identical aliquot of the sample in the NMR tube was separately titrated using the Ca^{2+}-specific electrode. The binding data were then best-fit to a single-site binding model, with the assumption that each carboxylate group coordinates a single Ca^{2+}. While this assumption is likely an oversimplification of the Ca^{2+}-carboxylate binding interactions predicted from the crystal structure, the data obtained did not allow for a more complex analysis. Thus, we chose to present the most simple interpretation of the data.

Titrations of the [^{13}C] chemical shifts of each selectively labeled Gla residue as a function of the $[Ca^{2+}]_{free}$ are shown in Figures 2−6. Different types of titration behavior are observed, consisting of both Ca^{2+}-induced increases and decreases in chemical shifts of the $^{13}COOH$ groups. This is probably due to mechanisms involving both shielding/deshielding and conformational changes in the polypeptide, all contributing to the [^{13}C] chemical shift effects observed. $K_{d,app}$ values characteristic of each Ca^{2+}−Gla carboxylate interaction were calculated on the basis of a single-site binding model. This interpretation was sufficient for all Gla residues except for Gla^{16} (Figure 3, bottom), Gla^{25} (Figure 5, top), and Gla^{26} (Figure 5, bottom), which demonstrated biphasic titration behavior. Thus, the data for these two residues were fit to a two-site noninteracting binding model as the simplest interpretation, since no data exist for any other construction, and since the spread of data points that is necessitated by this fairly insensitive method was not amenable to evaluation of the possibility of binding cooperativity.

The $K_{d,app}$ values for each Ca^{2+}−Gla carboxylate interaction are listed in Table 1.

DISCUSSION

As is the case with other vitamin K-dependent proteins, human PC and APC contain a variety of Ca^{2+} binding sites that play a role in their functional properties. We found that

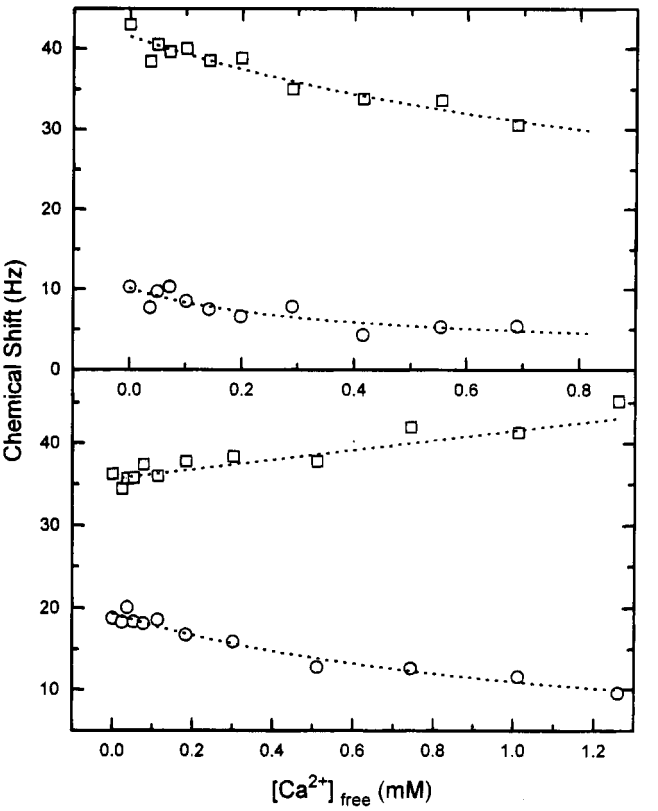

FIGURE 2: Titration of the chemical shifts of [di-γ-^{13}COOH]-enriched Gla residues as a function of $[Ca^{2+}]_{free}$. The buffer was sodium borate and 100 mM NaCl, 7.4 at 25 °C. Top: [di-γ-^{13}COOH-Gla^{6}]-GD/HS. Bottom: [di-γ-^{13}COOH-Gla^{7}]-GD/HS. The absolute values of the chemical shifts on the ordinate axis are arbitrarily chosen so that the differences as a function of $[Ca^{2+}]$ are emphasized.

human PC possesses at least three to four relatively tight Ca^{2+} binding sites (K_d = 0.08−0.12 mM), as well as a weaker set of at least ten Ca^{2+} sites (K_d = 0.5−1.5 mM) (Colpitts & Castellino, 1994). These cation sites are distributed over several regions of PC. At least one to two of the tight (K_d = 0.06−0.22 mM) and at least seven of the weak (K_d = 0.8−1.1 mM) Ca^{2+} sites have been shown to exist in the GD (Colpitts & Castellino, 1994). The molecular details of Ca^{2+} binding to the GD of a similar protein have become more clear with the publication of the X-ray crystal structure of the prothrombin fragment 1−Ca^{2+} complex (Soriano-Garcia et al., 1989, 1992). The basic features of this binding show that seven Ca^{2+} ions are coordinated in the GD, three of which are not solvated, with the other four hydrated to varying extents. Five Ca^{2+} ions are coordinated by Gla residues (PC numbering) 7 (6), 8 (7), 17 (16), 26 (25), and 27 (26). $Gla^{15(14)}$, $Gla^{20(19)}$, and $Gla^{21(20)}$ form a looser array with Ca^{2+}. Twenty-four of the 36 carboxylate oxygen atoms are located at distances from Ca^{2+} that suggest direct interactions with this cation, with $Gla^{17(16)}$ and $Gla^{27(26)}$ being completely buried. Several coordination types (unidentate, bidentate, and malonate type) have been implicated in stabilization of the Ca^{2+}−carboxylate interactions.

In addition to the above, one to two Ca^{2+} binding sites have been located outside of the GD, one of which (K_d = 0.1 mM) has been found to exist in the EGF-1 domain of PC (Ohlin et al., 1988). The presence of such a site is consistent with similar findings for EGF-1 regions of other vitamin K-dependent proteins, such as factor IX (Handford

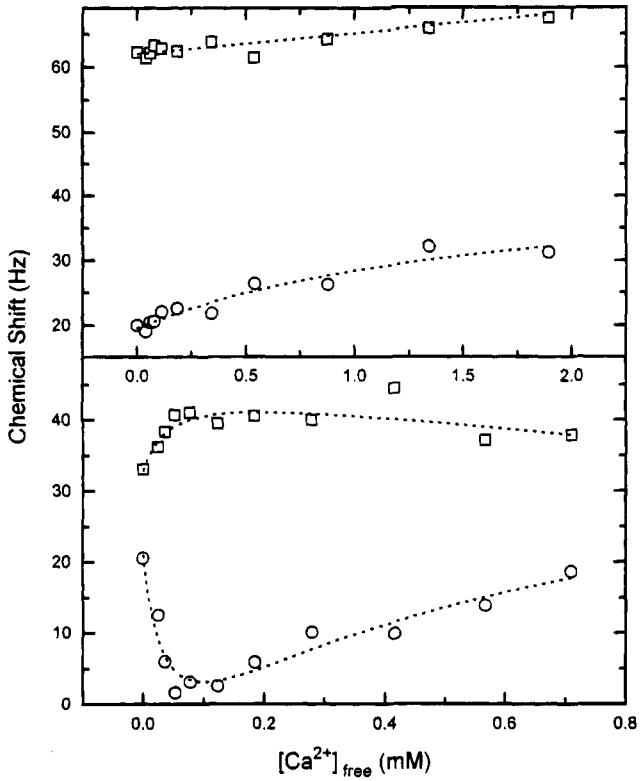

FIGURE 3: As in Figure 2 except that the peptides are (top) [di-γ-^{13}COOH-Gla^{14}]-GD/HS and (bottom) [di-γ-^{13}COOH-Gla^{16}]-GD/HS.

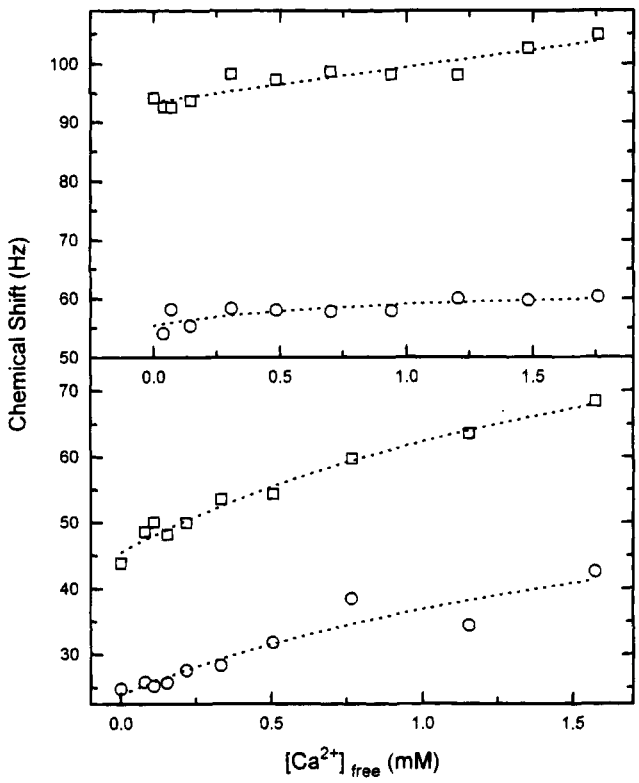

FIGURE 4: As in Figure 2 except that the peptides are (top) [di-γ-^{13}COOH-Gla^{19}]-GD/HS and (bottom) [di-γ-^{13}COOH-Gla^{20}]-GD/HS.

et al., 1990; Astermark et al., 1991) and factor X (Ohlin et al., 1988; Selander-Sunnerhagen et al., 1992), particularly since PC contains the side-chain residues in its EGF-1 region, *viz.*, D^{46}, D^{48}, Q^{49}, and D^{71}, that have been found to be important to Ca^{2+} binding to this same region in factor IX and factor X (Handford et al., 1991). Another high-affinity

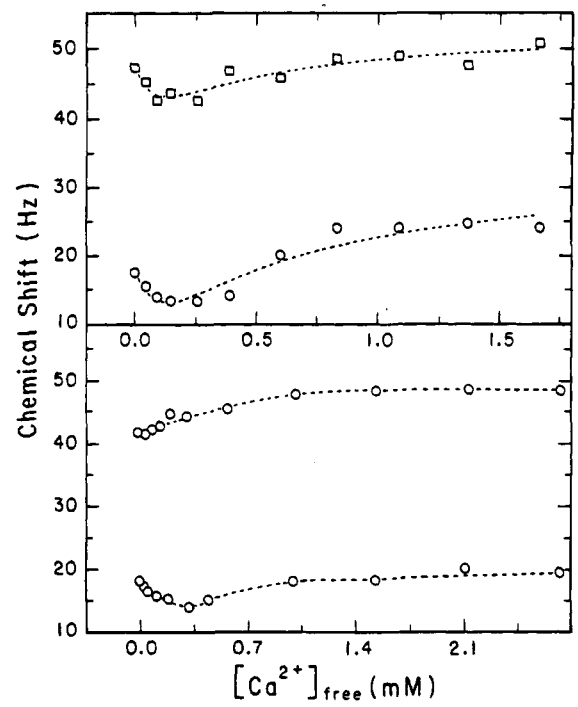

FIGURE 5: As in Figure 2 except that the peptides are (top) [di-γ-^{13}COOH-Gla^{25}]-GD/HS and (bottom) [di-γ-^{13}COOH-Gla^{26}]-GD/HS.

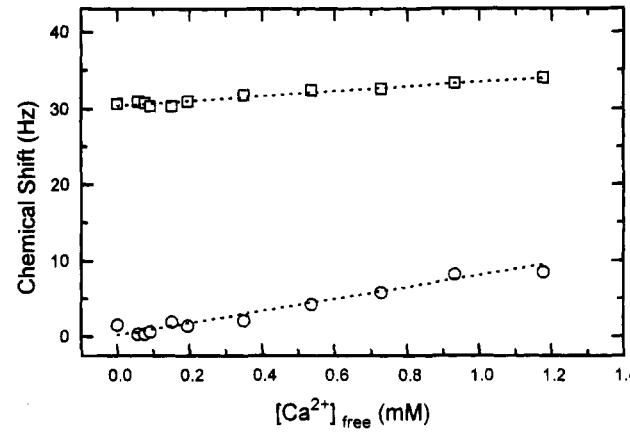

FIGURE 6: As in Figure 2 except that the peptide is [di-γ-^{13}COOH-Gla^{29}]-GD/HS.

Ca^{2+} binding site has been discovered outside of the Gla and EGF regions of PC (Rezaie et al., 1992). This latter site may be present in its protease domain, since other proteins of this type contain such a cation site (Bajaj et al., 1992; Wildgoose et al., 1993) in a region homologous to the known cation binding site in trypsin (Bode & Schwager, 1975) and elastase (Meyer et al., 1988).

The functions of these multiple Ca^{2+} binding sites in PC are varied. Ca^{2+} binding to the GD has been postulated to be responsible for production of a conformational alteration that is requisite to PL binding (Nelsestuen, 1976; Borowski et al., 1986a). This, in turn, is necessary for the biological activity of APC (Esmon et al., 1983). Investigations with r-PC and r-APC containing mutations at Gla residues suggest that Gla^{16} and Gla^{26} play uniformly important roles in allowing the proper Ca^{2+}-induced conformation, in PL binding, and in the biological activity of APTT (Table 1). On the other hand, Gla^{14} and Gla^{19} appear unimportant to many of these same properties. The remaining Gla residues have variable effects on these properties of PC and APC

Table 1: Calcium-Dependent Properties of the γ-Carboxyglutamic Acid Domain of Human Protein C and Activated Protein C

	binding studies[a]				mutational analysis[b]			
	$[K_{d,app}]_1$ (mM)		$[K_{d,app}]_2$ (mM)		MAb[d]	APTT[e]	PL binding[f]	
[^{13}C] at[c]	γ_1	γ_2	γ_1	γ_2	$[C_{50,Ca}]$ (mM)	%	$[C_{50,Ca}]$ (mM)	$[C_{50,PC}]$ (mM)
wt[g]					3.6	100	1.2	0.3
Gla^6	1.3	0.4			nb[h]	86	1.5	1.2
Gla^7	1.2	>5			nb[h]	6	8.2	0.4
Gla^{14}	1.7	>5			11	92	1.4	0.2
Gla^{16}	0.04	0.04	>5	0.50	>20	<2	>10	1.2
Gla^{19}	1.1	>5			14	80	1.1	1.8
Gla^{20}	2.4	2.2			>20	<2	>10	1.1
Gla^{25}	0.14	0.18	0.28	0.38	2	24	0.5	0.2
Gla^{26}	0.20	0.57	0.59		>20	<2	<10	1.6
Gla^{29}	>5	>5			17	9	2.8	0.2

[a] Binding to synthetic 47-mer peptides. [b] The mutations to Asp at the Gla residues in column 1. The data are from Zhang and Castellino (1992, 1993) and Zhang et al. (1992). [c] The position of the [di-γ-^{13}COOH]-Gla. [d] The Ca^{2+} concentration required for binding 50% of the PC to a MAb directed to the Ca^{2+}-dependent conformation of the GD (Wakabayashi et al., 1986). [e] The APTT activity of the mutant r-APC relative to the wt enzyme. [f] The Ca^{2+} concentration required for binding of 50% of the PC to PL vesicles ($[C_{50,Ca}$-PL]) or the PC concentration required for binding of 50% of the PC molecules to PL at a Ca^{2+} concentration of 20 mM ($[C_{50,PC}$-PL]). [g] Wild-type r-PC or r-APC. [h] These Gla residues are present at the epitope for the MAb used, and MAb binding does not occur in their absence.

(Zhang & Castellino, 1992, 1993; Zhang et al., 1992). In order to attempt to correlate structure−function properties of r-PC and r-APC with discrete Ca^{2+} binding, we required an approach that would allow determination of the roles of individual Gla residues in Ca^{2+} binding to the entire GD of PC. With the finding that a synthetic 47-mer peptide, containing the GD plus the trailing HS, provided an excellent model for the binding of Ca^{2+} to the GD of PC (Colpitts & Castellino, 1994), combined with the discovery that the chemical shifts of [di-γ-^{13}COOH]-labeled Gla were sensitive to the presence of Ca^{2+} (Colpitts & Castellino, 1993, 1994), selectively [di-γ-^{13}COOH]-labeled peptides provided the opportunity to study each Gla residue separately in the intact GD.

Using this approach, the data of Figures 2−6 (summarized in Table 1) allow us to conclude that Gla^{16} provides the tightest binding sites for Ca^{2+} in the GD of PC, with Gla^6, Gla^{25}, and Gla^{26} contributing to a class of binding sites possessing slightly weaker affinity for Ca^{2+} (<1.0 mM). These, plus the remaining Gla residues, compose the weak class of Ca^{2+} binding sites in the GD of PC. Some clear correlations of these Ca^{2+} binding data with results from site-directed mutagenesis Gla residues (summarized in Table 1) can be made with the aid of a model of the GD−Ca^{2+} complex of PC. Such a model is provided in Figure 7, which was generated on the basis of the coordinates derived from the crystal structure of this same region of bovine prothrombin.

Of the four Gla residues that display high affinity for Ca^{2+}, Gla^{16} and Gla^{26}, when subjected to conservative mutagenesis (to Asp residues), substantially affect the ability of the GD of PC to adopt its Ca^{2+}-dependent conformation and to productively bind to PL vesicles (Zhang & Castellino, 1992, 1993). These same mutations also result in loss of APTT activity of APC (Zhang et al., 1992). The molecular basis for these effects likely lies in the proposal that Gla^{16} and Gla^{26} coordinate primarily to internal Ca^{2+} ions (Ca2, Ca3, and Ca4, Figure 7) that are needed for stabilization of the Ca^{2+}-dependent conformation. Their effects on functionally relevant PL binding are probably indirectly related to the inability of the GD to assume the proper conformation needed for competent PL binding.

Another strong Ca^{2+} locus exists at Gla^{25}. Both carboxylates of this residue are involved in higher affinity binding of this cation (Table 1), which is likely Ca1. Previous studies have indicated that a r-PC containing a mutation to Asp at this position does not substantially affect PL binding or structural recognition by a MAb that is directed to the Ca^{2+}-dependent conformation of the GD (Zhang & Castellino, 1992, 1993). However, the anticoagulant activity of the mutant r-APC was reduced to 24% of that of wtr-APC (Zhang et al., 1992). As a previous possible explanation of these data, we proposed that Gla^{25} may be responsible for stabilizing binding of a Ca^{2+} ion (Ca1) that is required for orienting the GD on the PL surface such that the active site of APC is aligned with the APC-sensitive cleavage sites of its physiological substrates, fVa and fVIIIa. Elimination of the tight coordination sites provided by Gla^{25} to Ca1 should drastically weaken, or perhaps eliminate, binding of Ca1 to PC and APC. Our previous assumption that Ca1 binding to the GD of PC could be disrupted by alterations in the integrity of Gla^{25} is confirmed by the current data, which show that Gla^{25} does contribute a strong protein site for Ca1.

The final Gla residue that contributes to a high-affinity Ca^{2+} site is Gla^6. However, only one carboxylate of this residue is involved in Ca^{2+} binding. When Gla^6 is mutated to Asp in r-PC and r-APC, the Ca^{2+}-dependent properties associated with the GD are largely unaffected (Zhang & Castellino, 1992, 1993; Zhang et al., 1992). However, when this residue is mutated to Val, APTT activity is lost, indicating that the Ca^{2+} binding properties that have been affected are significant to the function of the GD (Christiansen et al., 1994). Since this residue mainly provides tight binding sites to Ca5 (Figure 7), it appears as though this binding is crucial to function. The fact that the conversion to the Asp^6 mutant does not result in deleterious effects on functional properties of the GD can be interpreted to indicate that the carboxylate of Asp at this location serves a role similar to that of the lone carboxylate of Gla^6.

Considering the remaining Gla residues, Gla^{14} and Gla^{19} possess low affinities for Ca^{2+}, and conservative mutations to Asp (Zhang & Castellino, 1992, 1993; Zhang et al., 1992) or radical mutations to Val at these positions result in mutants that possess normal Ca^{2+}-dependent GD-related properties

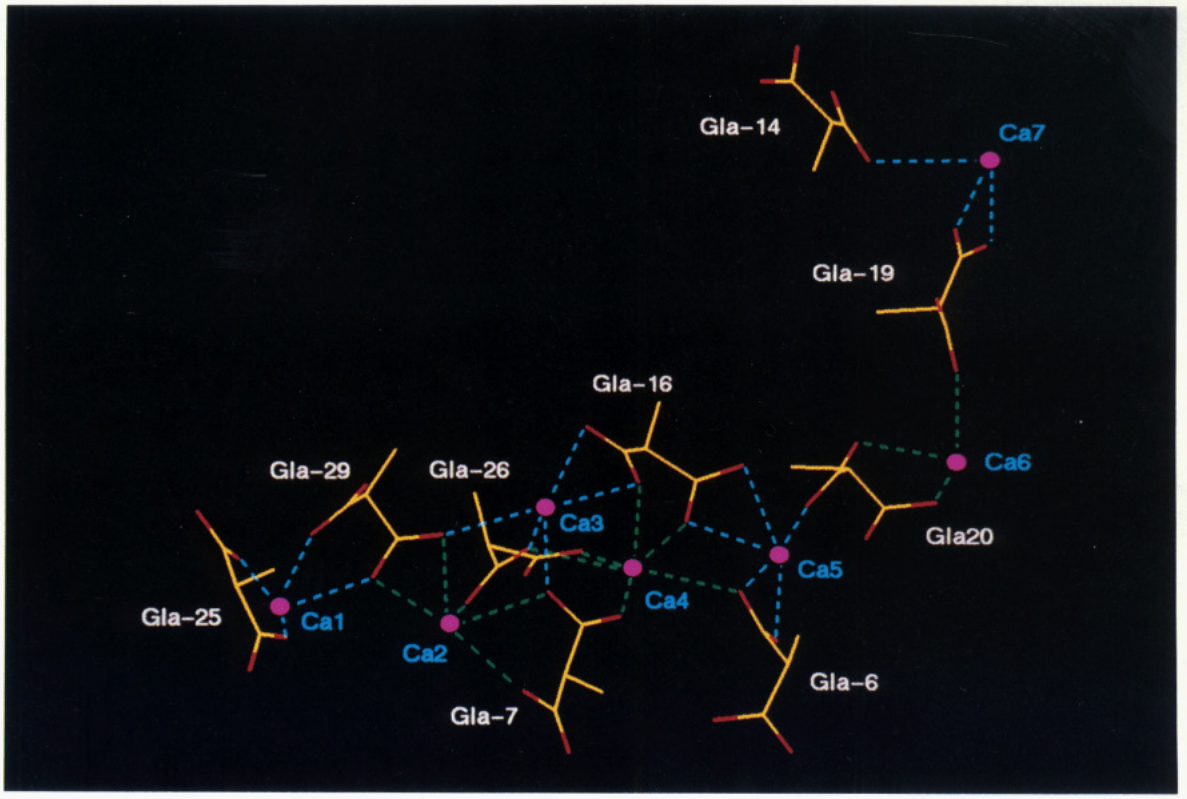

FIGURE 7: Model of the three-dimensional structure of the Gla domain and helical stack of PC based on the crystal coordinates of bovine prothrombin fragment 1. The locations of Gla residues and their proposed coordination sites to Ca^{2+} are shown. The atoms involved in Ca^{2+}–O coordination are based on distance measurements in the model and have been assigned when Ca^{2+} and O atoms were located between 2.2 and 3.1 Å from each other. Ca^{2+} is in magenta. Gla residues shown from the β-carbon are colored yellow, with their oxygen atoms in red.

(Christiansen et al., 1994). In the model of Figure 7, these two particular Gla residues coordinate to the surface-exposed Ca7. From these data, we conclude that Ca7 is neither important to adoption of the Ca^{2+}-induced conformation of the GD (Zhang & Castellino, 1992) nor important for functional PL binding (Zhang & Castellino, 1993).

Gla^{19}, along with Gla^{20}, contributes coordination sites to Ca6, and from the data of Table 1, this is predicted to be one of the weak-affinity Ca^{2+} ions. We concluded previously that this surface-exposed Ca^{2+} was a major contributor to PL binding, and consequent APTT activity, but played a more minor role in induction of the Gla-dependent conformation of the GD (Zhang & Castellino, 1992, 1993; Zhang et al., 1992). These effects could be explained by proposing that the coordination locus provided by Gla^{20} is essential to the stability of Ca6 binding and that its disruption affects functional PL binding that is needed for productive Ca^{2+}–PL interactions. In addition, Gla^{20} also coordinates an interior Ca^{2+} (Ca5) which is structurally important. Mutation of Gla^{20} to Asp disrupts the conformational integrity of the GD as evidenced by loss of monoclonal antibody recognition (Zhang & Castellino, 1992). That Gla^{20} has only a weak affinity for Ca^{2+} in this peptide supports the observation that the Ca^{2+}-induced conformational change in the GD of PC can be correlated with occupancy of the weak sites. The interesting note here is that Gla^{20} then is the only strictly low Ca^{2+} affinity Gla residue shown to be essential to the Ca^{2+}-dependent properties of the GD of PC.

The fact that conservative mutation (to Asp) of Gla^{7} does not significantly affect the ability of the GD to adopt its Ca^{2+}-dependent conformation is consistent with its provision of only a weak coordination locus for the less solvent-exposed cations, Ca3 and Ca4, which are otherwise tightly bound to Gla^{16} (Table 1). On the other hand, the observation that this same mutation in Gla^{7} results in nonproductive PL binding along with a large activity loss of the mutant r-APC shows that the surface-exposed Ca2 is also involved with the second, direct stage of functional PL binding (Zhang & Castellino, 1992, 1993; Zhang et al., 1992). Since Ca2 is only coordinated through weak sites provided by Gla^{7} and Gla^{29}, it would be expected that loss of Gla^{7} would have a greater effect on Ca2-related functions than those of Ca3 and Ca4. A similar argument can be forwarded for the similar effects of a conservative Gla^{29} mutation on PL binding and APC activity (Zhang & Castellino, 1992, 1993; Zhang et al., 1992).

Among the major conclusions drawn from this work is that the conformational alterations that produce a biologically functional form of PC and APC correlate well with the weak class of binding sites. This was generally demonstrated by showing that both PC (Zhang & Castellino, 1992, 1993) and Gla domain-derived peptides [this work and Colpitts and Castellino (1994)] require >1 mM Ca^{2+} for productive PL binding. Thus, while some of the high-affinity sites may be needed for inducing a conformation resulting in functional PL binding, additional binding of Ca^{2+} to low-affinity divalent cation sites may be required as well, and these latter events can be probed by titrations with Ca^{2+} of the divalent cation-dependent fluorescence quenching of PC, MAb binding, PL binding, circular dichroism changes, and biological activity.

The investigation reported herein is a significant advance in our understanding of discrete structure−function relationships of the GD of PC and APC in that we document the ability to examine Ca^{2+} binding to each Gla residue separately in a protein wherein nine Gla residues coordinate Ca^{2+}. This now provides an excellent opportunity to examine the functional effects of mutations of the GD in intact PC on the ability of any single Gla residue to coordinate Ca^{2+} and opens very important avenues that will allow us to understand structure−function relationships of this important group of proteins using the entire range of modern chemical and biological methodology.

REFERENCES

Amphlett, G. W., Byrne, R., & Castellino, F. J. (1979) *J. Biol. Chem. 254,* 6333−6336.

Amphlett, G. W., Kisiel, W., & Castellino, F. J. (1981a) *Biochemistry 20,* 2156−2161.

Amphlett, G. W., Kisiel, W., & Castellino, F. J. (1981b) *Arch. Biochem. Biophys. 208,* 576−585.

Astermark, J., Bjork, I., Ohlin, A.-K., & Stenflo, J. (1991) *J. Biol. Chem. 266,* 2430−2437.

Bajaj, S. P. (1982) *J. Biol. Chem. 257,* 4127−4132.

Bajaj, S. P., Butkowski, R. J., & Mann, K. G. (1975) *J. Biol. Chem. 250,* 2150−2156.

Bajaj, S. P., Sabharwal, A. K., Gorka, J., & Birktoft, J. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89,* 152−156.

Beals, J. M., & Castellino, F. J. (1986) *Biochem. J. 236,* 861−869.

Bode, W., & Schwager, P. (1975) *FEBS Lett. 56,* 139−143.

Borowski, M., Furie, B. C., Bauminger, S., & Furie, B. (1986a) *J. Biol. Chem. 261,* 14969−14975.

Borowski, M., Furie, B. C., & Furie, B. (1986b) *J. Biol. Chem. 261,* 1624−1628.

Chibber, B. A. K., Urano, S., & Castellino, F. J. (1990) *Int. J. Pep. Protein Res. 35,* 73−80.

Christiansen, W. T., Tulinsky, A., & Castellino, F. J. (1994) *Biochemistry* (in press).

Church, W. R., Bhushan, F. H., Mann, K. G., & Bovill, E. G. (1989a) *Blood 74,* 2418−2425.

Church, W. R., Boulanger, L. L., Messier, T. L., & Mann, K. G. (1989b) *J. Biol. Chem. 264,* 17882−17887.

Colpitts, T. L., & Castellino, F. J. (1993) *Int. J. Pep. Protein Res. 41,* 567−575.

Colpitts, T. L., & Castellino, F. J. (1994) *Biochemistry 33,* 3501−3508.

Esmon, N. L., DeBault, L. E., & Esmon, C. T. (1983) *J. Biol. Chem. 258,* 5548−5553.

Handford, P. A., Baron, M., Mayhew, M., Willis, A., Beesley, T., Brownlee, G. G., & Campbell, I. D. (1990) *EMBO J. 9,* 475−480.

Handford, P. A., Mayhew, M., Baron, M., Winship, P. R., Campbell, I. D., & Brownlee, G. G. (1991) *Nature 351,* 164−167.

Henriksen, R. A., & Jackson, C. M. (1975) *Arch. Biochem. Biophys. 170,* 149−159.

Keyt, B., Furie, B. C., & Furie, B. (1982) *J. Biol. Chem. 257,* 8687−8695.

Kisiel, W., Canfield, W. M., Ericsson, L. H., & Davie, E. W. (1977) *Biochemistry 16,* 5824−5831.

Liebman, H. A. (1993) *Eur. J. Biochem. 212,* 339−345.

Liebman, H. A., Furie, B. C., & Furie, B. (1987) *J. Biol. Chem. 262,* 7605−7612.

Lowry, D. H., & Lopez, J. A. (1946) *J. Biol. Chem. 162,* 421−428.

Marki, W., Oppliger, M., & Schwyzer, R. (1977) *Helv. Chim. Acta 60,* 807−815.

Meyer, E., Cole, G., Radhakrishnan, R., & Epp, O. (1988) *Acta Crystallogr., Sect. B: Struct. Crystallogr. 44,* 26−39.

Nelsestuen, G. L. (1976) *J. Biol. Chem. 251,* 5648−5656.

Nelsestuen, G. L., & Suttie, J. W. (1972) *Biochemistry 11,* 4961−4964.

Ohlin, A.-K., Linse, S., & Stenflo, J. (1988) *J. Biol. Chem. 263,* 7411−7417.

Prendergast, F. G., & Mann, K. G. (1977) *J. Biol. Chem. 252,* 840−850.

Rezaie, A. R., Esmon, N. L., & Esmon, C. T. (1992) *J. Biol. Chem. 267,* 11701−11704.

Selander-Sunnerhagen, M., Ullner, M., Persson, E., Teleman, O., Stenflo, J., & Drakenberg, T. (1992) *J. Biol. Chem. 267,* 19642−19649.

Soriano-Garcia, M., Park, C. H., Tulinsky, A., Ravichandran, K. G., & Skrzypczak-Jankun, E. (1989) *Biochemistry 28,* 6805−6810.

Soriano-Garcia, M., Padmanabhan, K., deVos, A. M., & Tulinsky, A. (1992) *Biochemistry 31,* 2554−2566.

Strickland, D., & Castellino, F. J. (1980) *Arch. Biochem. Biophys. 199,* 61−66.

Tulinsky, A., Park, C. H., Boryeu, M., & Llinas, M. (1988) *Proteins: Struct., Funct., Genet. 3,* 85−96.

Vehar, G. A., & Davie, E. W. (1980) *Biochemistry 19,* 401−410.

Wakabayashi, K., Sakata, Y., & Aoki, N. (1986) *J. Biol. Chem. 261,* 11097−11105.

Wildgoose, P., Foster, D., Schiodt, J., Wiberg, F. C., Birktoft, J. J., & Petersen, L. C. (1993) *Biochemistry 32,* 114−119.

Zhang, L., & Castellino, F. J. (1990) *Biochemistry 29,* 10828−10834.

Zhang, L., & Castellino, F. J. (1992) *J. Biol. Chem. 267,* 26078−26084.

Zhang, L., & Castellino, F. J. (1993) *J. Biol. Chem. 268,* 12040−12045.

Zhang, L., Jhingan, A., & Castellino, F. J. (1992) *Blood 80,* 942−952.

BI941709J